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Minocycline and Pyrrolidine Dithiocarbamate Attenuate Hypertension via Suppressing Activation of Microglia in the Hypothalamic Paraventricular Nucleus

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Proinflammatory cytokines, reactive oxygen species and imbalance of neurotransmitters are involved in the pathophysiology of angiotensin II-induced hypertension. The hypothalamic paraventricular nucleus (PVN) plays a vital role in hypertension. Evidences show that microglia are activated and release proinflammatory cytokines in angiocardiopathy. We hypothesized that angiotensin II induces PVN microglial activation, and the activated PVN microglia release proinflammatory cytokines and cause oxidative stress through nuclear factor-kappa B (NF-κB) pathway, which contributes to sympathetic overactivity and hypertension. Male Sprague-Dawley rats (weight 275-300 g) were infused with angiotensin II to induce hypertension. Then, rats were treated with bilateral PVN infusion of microglial activation inhibitor minocycline, NF-κB activation inhibitor pyrrolidine dithiocarbamate or vehicle for 4 weeks. When compared to control groups, angiotensin II-induced hypertensive rats had higher mean arterial pressure, PVN proinflammatory cytokines, and imbalance of neurotransmitters, accompanied with PVN activated microglia. These rats also had more PVN gp91^{phox} (source of reactive oxygen species production), and NF-κB p65. Bilateral PVN infusion of minocycline or pyrrolidine dithiocarbamate partly or completely ameliorated these changes. This study indicates that angiotensin II-induced hypertensive rats have more activated microglia in PVN, and activated PVN microglia release proinflammatory cytokines and result in oxidative stress, which contributes to sympathoexcitation and hypertensive response. Suppression of activated PVN microglia by minocycline or pyrrolidine dithiocarbamate attenuates inflammation and oxidative stress, and improves angiotensin II-induced hypertension, which indicates that activated microglia promote hypertension through activated NF-κB. The findings may offer hypertension new strategies.

Keywords: hypertension; hypothalamic paraventricular nucleus; microglia; minocycline; pyrrolidine dithiocarbamate
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Introduction

Hypertension is one of the most common angiocardiopathy associated with heredity and environment, which can cause damage to target organs such as heart, brain, kidney, peripheral blood vessels and eye ground (Cai et al. 2020). The hypothalamic paraventricular nucleus (PVN) is acknowledged as cardiovascular integrative center, which devoted to the augmented sympathetic activity in hypertension (Yu et al. 2021b). Inflammation and oxidative stress are closely related to the sympathoexcitation and the complex pathogenesis of hypertension. Relationship between hypertension and inflammation has been demonstrated in experimental models of hypertension where tissue expression and plasma concentrations of inflammatory mediators are elevated (Yu et al. 2021a, b). Our researches have indicated that sympathoexcitation in hypertension is partly due to the increase of proinflammatory cytokines in the PVN, and blocking the production of proinflammatory cytokines downregulates the sympathetic overactivity (Yu et al. 2019). Recently, large amount of evidence indicates that reactive oxygen species (ROS) is involved in the hypertensive activities. Our previous studies proved that subunits of nicotinamide adenine dinucleotide phosphate [NAD(P)H] oxidase ($gp91^{phox}$, source of ROS production) was elevated in the PVN of hypertension (Yu et al. 2021a, b; Gao et al. 2021). It has been also documented that sympathetic overactivity and pathophysiology of hypertension are associated with imbalance of neurotransmitters including norepinephrine (NE), glutamate (Glu) and γ-aminobutyric acid (GABA) (Yi et al. 2016; Yu et al. 2021a). NE and Glu act as excitatory neurotransmitters to enhance the sympathetic nerve activity, while GABA acts as an inhibitory neurotransmitter to attenuate the sympathetic activity. However, the central mechanism is not known.

The nuclear factor-kappa B (NF-κB) family of eukaryotic transcription factors is in PVN neurons and plays a vital part in inflammation (Kang et al. 2014a). Activated NF-κB is the major regulator facilitating the synthesis of several different injury-responsive cytokines in neurons, including proinflammatory cytokines (Kang et al. 2014a). Pyrrolidine dithiocarbamate (PDTC) is an inhibitor of NF-κB activation in a variety of cells. Oxidative stress and sympathoexcitation associated with NAD(P)H oxidase were also demonstrated to be caused by activated NF-κB in hypertension-related cardiovascular diseases (Queisser and Schupp 2012; Kang et al. 2014a). Prostaglandin E_2 (PGE_2), which comes into being by arachidic acid produced by cyclooxygenase (COX) enzyme metabolism (namely COX-1 and COX-2), are inflammatory medium. PGE_2 can go across the blood-brain barrier, and induce elevation of proinflammatory cytokines in the central nervous system (CNS). PGE_2 itself also participated in the sympathoexcitation (Kang et al. 2008b). Angiotensin II (ANG II) is related to the pathological physiology of hypertension. ANG II binds to ANG II type 1 receptor and consequently promotes ROS generation (Chen et al. 2019). One study in our laboratory found that the treatment with the ANG II type 1 receptor antagonist losartan or the superoxide dismutase mimetic tempol decreased proinflammatory cytokines in the PVN in hypertensive rats induced by ANG II, which is a potential activator of sympathetic drive (Kang et al. 2008a).

Studies have implicated that hypertension induced by ANG II is in relation to PVN activated microglia (Biancardi et al. 2016). Microglia are considered to produce deleterious proinflammatory medium to result in the start of inflammation in many cerebral diseases (Pajares et al. 2020). In the central nervous system (CNS), microglia are activated in neuro-inflammatory events and neurodegenerative diseases (Salter and Stevens 2017; Pajares et al. 2020), and contribute significantly to the release of PGE_2 and proinflammatory cytokines (Subedi et al. 2019). PGE_2 are largely produced by activated microglia during brain inflammation, and inhibition of microglia activation has been shown to improve several neurodegenerative diseases (Pajares et al. 2020). As primary immunological effector cells of CNS, microglia respond to injury at a very early stage, even with minor pathological changes in the CNS. This type of glial cells is ubiquitously distributed in the CNS, including the PVN (Wang et al. 2019). Resting microglia show a downregulated immunophenotype, and their ability to respond selectively to neurotransmission allows them reacting rapidly in event of pathological disturbances, and may release several mediator substances including proinflammatory cytokines, like interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). Besides, COX-2 is upregulated to preferentially catalyze the formation of PGE_2 in activated microglia (Subedi et al. 2019). Minocycline (MIN) is a tetracycline, which can inhibit the activation of microglia in the brain. The present study was designed to investigate whether activated microglia in the PVN contribute to sympathoexcitation in hypertension through NF-κB pathway affecting proinflammatory cytokines, ROS, PGE_2 and the imbalance of neurotransmitters. In this study, both MIN and PDTC were used to observe the mechanisms of microglia in the PVN in hypertension.

Materials and Methods

Animals

Male adult Sprague-Dawley rats (275-300 g) were applied in this experiment. The rats were accommodated in a room with constant temperature and humidity and 12-h light-dark cycle, and were ensured with standard rat chow and tap water. At the same time, all rats were maintained on a daily sodium intake of 8.7 g NaCl/kg (normal salt intake). Experiment was approved by the Experimental Animal Care Use Committees of Xi’an Jiaotong University and in compliance with Guide for the Care and Use of Laboratory Animals published by United States National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Experimental scheme

ANG II (Sigma, St. Louis, MO, USA) was infused intravenously at 0.6 μg/hr for 4 weeks by the Alzet osmotic minipump (DURECT, Cupertino, CA, USA) to develop hypertensive rats. Control rats was administrated with normal saline (NS, 0.3% NaCl). The rats were implanted with PVN cannulae to receive PDTC (5μg/hr, Sigma), MIN (5 µg/hr, Macklin, Shanghai, China) or vehicle (artificial cerebrospinal fluid, aCSF) separately for 4 weeks. All the doses are based on our prevenient research (Fisher and Paton 2012; Shen et al. 2018). At the end, blood and brain specimens were gathered for investigation. They were randomly divided into 6 groups based on the treatment they received: (1) NS + aCSF; (2) NS + PDTC; (3) NS + MIN; (4) ANG II + aCSF; (5) ANG II + PDTC; (6) ANG II + MIN.

Imbedding of venous and arterial catheters and bilateral PVN cannulae

Rats were anesthetized, and placed into a stereotaxic apparatus. Catheters were imbedded in femoral vein and then in vena cava for minipump infusion of ANG II, and in femoral artery and the abdominal aorta for arterial pressure measurement. The ends were tunneled subcutaneously to nape and anchored. Venous and arterial catheters were made up of polyethylene tubing (PE-10) and stainless steel pin. While still under anesthesia, each rat had a cannula implanted in the bilateral PVN, using stereotaxic coordinates (Fisher and Paton 2012; Yu et al. 2021b). The rats were admitted to restore for 3 days before experiment.

Drugs infused

Rats received bilateral PVN infusion of pyrrolidine dithiocarbamate (PDTC, 5μg/hr, Sigma), minocycline (MIN, 5 µg/hr, Macklin) or aCSF. The dosage of PDTC or MIN was chosen on the basis of former research (Fisher and Paton 2012; Shen et al. 2018).

Measurement of mean arterial pressure (MAP)

For the measurement of arterial pressure, surgical procedures were performed under anesthesia. Catheters were placed into the femoral artery and advanced into the abdominal aorta. The femoral artery cannula was flushed with 0.1 ml heparinized saline (50 units/ml) and connected to a pressure transducer attached to a digital BP monitor and a polygraph. MAP data were collected for 30 min between 8 and 11 a.m. and averaged (Qi et al. 2013).

Tissue sample assortment

The rats were decapitated under anesthesia to gather brain and blood tissue. Blood was collected from the left carotid artery and centrifuged at 3,000 rpm for 30 min. Then, plasma was removed to a clean 1.5 mL centrifuge tube and stored at −80°C for future analysis until assayed (Yu et al. 2021a). The procedure of tissue microdissection was previously described to separate the PVN (Agarwal et al. 2011). Briefly, the brain was sectioned serially in 300 mm increments from the bregma to lambda using a cryostat. The sections were transferred to coverslip and placed on a cold stage maintained at −10°C. The PVN was punched with the help of a stereotaxic atlas. Some of the microdissected PVN tissue was stored at −80°C until analyzed. The tissues were collected from both sides of the PVN of individual rat.

Immunofluorescence analysis

Immunofluorescence analysis was performed as noted before (Su et al. 2014). Rats were anesthetized and fixed with perfusion using 0.01M phosphate-buffered solution (PBS) into the left ventricle first and then with 4% paraformaldehyde. The brain samples were collected, soaked in 4% paraformaldehyde and then 30% sucrose. Tissue microdissection was used to separate the PVN tissue. Immunofluorescence was performed to observe CD11b (marker of activated microglia). The primary antibodies used were as follows: anti-CD11b (ab1211, Abcam, Cambridge, UK). Briefly, frozen brain sections were incubated with 0.3% Triton-X for 30 min at 37°C, and primary antibody at 4°C overnight. After being washed with PBS, the sections were incubated with corresponding secondary antibody for 120 min at 37°C, and then covered with coverslips and imaged using Nikon microscopy.

Biochemical assays

NE, IL-1β and ANG II in plasma, PGE_2 in cerebrospinal fluid (CSF), and TNF-α and IL-1β in PVN were quantified by ELISA kits (Biosource International, Camarillo, CA, USA) (Yu et al. 2021a). According to the manufacturer's descriptions, the standards or sample diluents were added in the appropriate well of microtiter plate precoated with specific antibodies and incubated. Conjugate was added and incubated at 37°C for 1 h and then washed. The reactions were stopped with stop solution using a microtiter plate reader (MK3, Thermo Fisher Scientific, Waltham, MA, USA). The PGE_2 in CSF was measured by a PGE_2 Express EIA Kit from (Cayman Chemical, Ann Arbor, MI, USA) following the procedures detailed in the instructions, with a detection limit of 7.8 pg/mL (Malvar et al. 2011). PVN NF-κB activity was assessed by the activity of p65 in the PVN nuclear fraction using the NF-κB p65 active ELISA kit (Active Motif, Carlsbad, CA, USA). Nuclear extracts were prepared and a sandwich ELISA method was performed according to the manufacturer's protocol. This procedure yields 0.15 to 0.25 mg of PVN tissue nuclear extract at 3 to 5 mg/ml. Active ELISA detection levels for NF-κB p65 from nuclear extracts were 0.5 to 1 μg per well at 450 nm (Qi et al. 2019).

High performance liquid chromatography (HPLC)

NE, glutamate (Glu), GABA in the PVN were detected by HPLC as shown before (Kang et al. 2011). PVN Glu and GABA were measured using HPLC with electrochemical detection (ECD-300, Eicom Corporation, Kyoto, Japan)

and PVN NE concentration was measured using HPLC with electrochemical detection (HTEC-500, Eicom Corporation) as previously described (Kang et al. 2011).

Real-time PCR

Real-time PCR was done for gp91^{phox} mRNA in PVN as previously described (Yu et al. 2021a). Total RNA was extracted from the PVN using TRIzol following the manufacturer's guidance. cDNA was synthesized using PrimeScrpt RT Master Mix. Real-time PCR was then performed using the SYBR Premix Ex Taq II in a Mx3005P qPCR system (Agilent Technologies, Santa Clara, CA, USA). The primer sequence used in this study is: forward primer 5′-CTGCCAGTGTGTCGGAATCT-3′; and reverse primer 5′-TGTGAATGGCCGTGTGAAGT-3′.

Statistics

Data were expressed as mean ± standard error of the mean (SEM), and $P < 0.05$ was regarded as significant. One-way ANOVA followed by a post-hoc Tukey test was used to analyze data.

Results

PVN infusion of MIN or PDTC attenuated MAP

Compared with control rats, MAP ascended in ANG II-induced hypertensive rats. PVN administration of MIN or PDTC resulted in significant decline in MAP of ANG II-induced hypertensive rats (Fig. 1).

Effects of PVN infusion of PDTC on PVN microglia

Immunofluorescence discovered increased PVN activated microglia in ANG II-induced hypertensive rats. Activated microglia were detected with retraction of processes (a stubby appearance) by CD11b which increases (Dworak et al. 2012). ANG II-induced hypertensive rats had more activated microglia in PVN when compared to control rats (Fig. 2). PDTC supplement decreased activated microglia in PVN of hypertensive rats (Fig. 2).

PVN administration of MIN or PDTC attenuated CSF PGE_2

CSF PGE_2 was increased in ANG II-induced hypertensive rats when compared to control ones. PVN administration of PDTC or MIN attenuated CSF PGE_2 in ANG II-induced hypertension (Fig. 3).

PVN administration of MIN or PDTC attenuated IL-1β and TNF-α in PVN

ELISA studies exhibited that TNF-α and IL-1β in PVN of ANG II-induced hypertensive rats were higher than those of control ones (Fig. 4). They decreased after treatment with MIN or PDTC compared with aCSF-treated ANG II-induced hypertensive rats (Fig. 4), but were still higher than those of control groups.

PVN administration of MIN or PDTC attenuated plasma NE, ANG II and IL-1β

Plasma IL-1β, NE and ANG II of ANG II-induced hypertensive rats elevated when compared with control rats (Fig. 5). It was demonstrated that PVN administration of PDTC or MIN attenuated increasement of plasma NE of ANG II-induced hypertensive rats significantly (Fig. 5A), and suppressed partly but not completely ANG II of ANG II-induced hypertensive rats (Fig. 5C). Plasma IL-1β of ANG II-induced hypertensive rats was not suppressed sig-

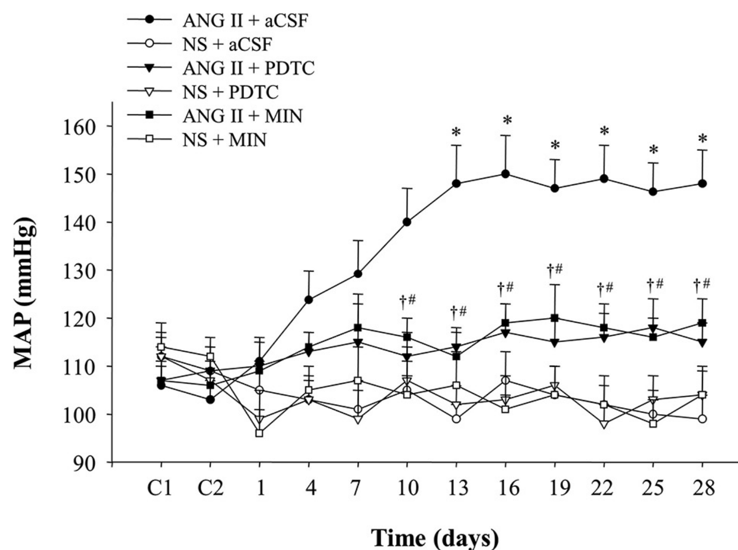

Fig. 1. Mean arterial pressure change before and after paraventricular nucleus infusion of minocycline or pyrrolidine dithiocarbamate. Effects of minocycline or pyrrolidine dithiocarbamate on mean arterial pressure of angiotensin II-induced hypertensive rats. *$P < 0.05$ vs. control rats (NS + aCSF or NS + MIN/PDTC); $^{\dagger}P < 0.05$ ANG II + MIN vs. ANG II + aCSF; $^{\#}P < 0.05$ ANG II + PDTC vs. ANG II + aCSF.
MAP, mean arterial pressure; NS, normal saline; aCSF, artificial cerebrospinal fluid; MIN, minocycline; PDTC, pyrrolidine dithiocarbamate; ANG II, angiotensin II.

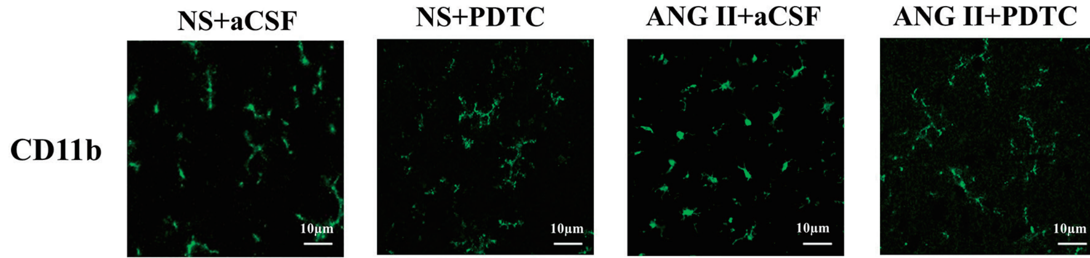

Fig. 2. Effects of paraventricular nucleus infusion of pyrrolidine dithiocarbamate on microglia in the paraventricular nucleus.
Immunofluorescence for the changes of the expression of CD11b in paraventricular nucleus after paraventricular nucleus infuse of pyrrolidine dithiocarbamate.
NS, normal saline; aCSF, artificial cerebrospinal fluid; PDTC, pyrrolidine dithiocarbamate; ANG II, angiotensin II.

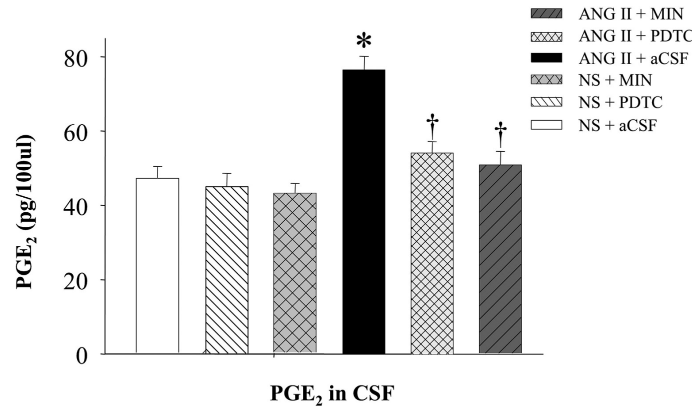

Fig. 3. Effects of paraventricular nucleus infusion of minocycline or pyrrolidine dithiocarbamate on prostaglandin E_2 in cerebrospinal fluid of angiotensin II-induced hypertensive rats.
Group data for prostaglandin E_2 (PGE_2) in CSF in different groups. *$P < 0.05$ vs. control rats (NS + aCSF or NS + MIN/PDTC); †$P < 0.05$ ANG II + MIN/PDTC vs. ANG II + aCSF.
PGE_2, prostaglandin E_2; CSF, cerebrospinal fluid; NS, normal saline; aCSF, artificial cerebrospinal fluid; MIN, minocycline; PDTC, pyrrolidine dithiocarbamate; ANG II, angiotensin II.

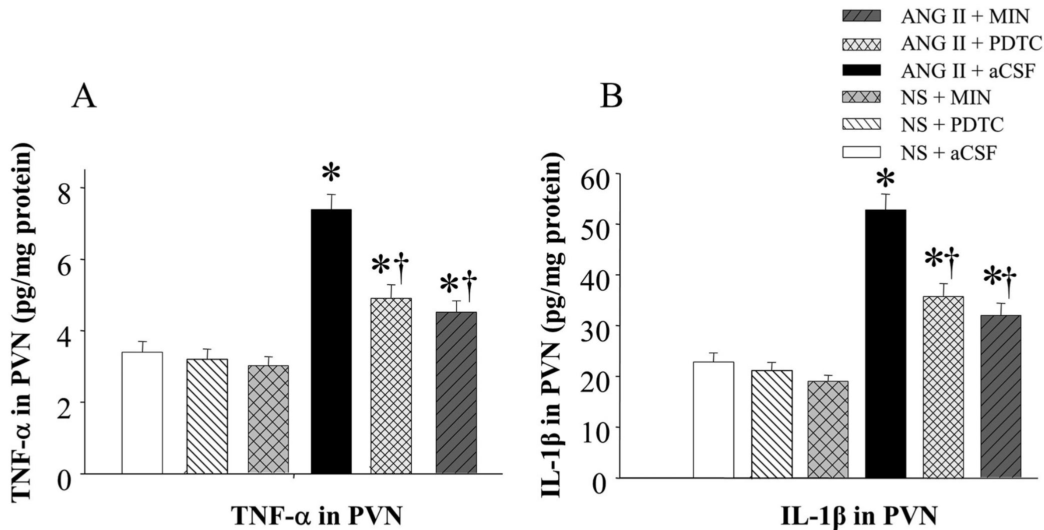

Fig. 4. Effects of paraventricular nucleus infusion of minocycline or pyrrolidine dithiocarbamate on tumor necrosis factor-α and interleukin-1β in the paraventricular nucleus.
A. Group data for tumor necrosis factor-α (TNF-α) expression in different groups measured by ELISA. *$P < 0.05$ vs. control rats (NS + aCSF or NS + MIN/PDTC); †$P < 0.05$ ANG II + MIN/PDTC vs. ANG II + aCSF. B. Group data for interleukin-1β (IL-1β) expression in different groups measured by ELISA. *$P < 0.05$ vs. control rats (NS + aCSF or NS + MIN/PDTC); †$P < 0.05$ ANG II + MIN/PDTC vs. ANG II + aCSF.
TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; PVN, paraventricular nucleus; NS, normal saline; aCSF, artificial cerebrospinal fluid; MIN, minocycline; PDTC, pyrrolidine dithiocarbamate; ANG II, angiotensin II.

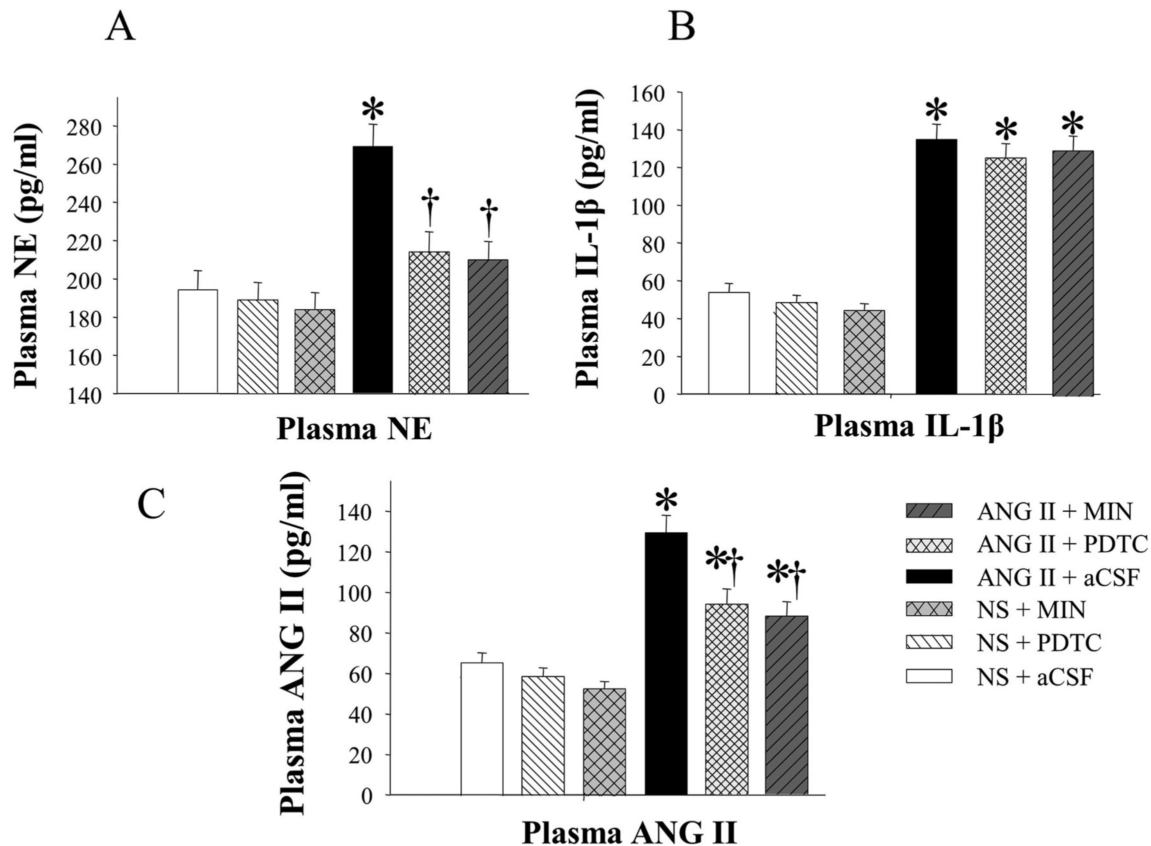

Fig. 5. Effects of paraventricular nucleus infusion of minocycline or pyrrolidine dithiocarbamate on plasma levels of norepinephrine, interleukin-1β and angiotensin II of angiotensin II-induced hypertensive rats.
A. Group data for plasma norepinephrine (NE) in different groups. *P < 0.05 vs. control rats (NS + aCSF or NS + MIN/PDTC); †P < 0.05 ANG II + MIN/PDTC vs. ANG II + aCSF. B. Group data for plasma interleukin-1β (IL-1β) in different groups. Paraventricular nucleus infusion of minocycline or pyrrolidine dithiocarbamate did not significantly alleviate plasma IL-1β induced by angiotensin II. *P < 0.05 vs. control rats (NS + aCSF or NS + MIN/PDTC). C. Group data for plasma angiotensin II (ANG II) in different groups. Paraventricular nucleus infusion of minocycline or pyrrolidine dithiocarbamate partly but not completely suppressed plasma angiotensin II of angiotensin II-induced hypertensive rats. *P < 0.05 vs. control rats (NS + aCSF or NS + MIN/PDTC); †P < 0.05 ANG II + MIN/PDTC vs. ANG II + aCSF.
NE, norepinephrine; IL-1β, interleukin-1β; ANG II, angiotensin II; NS, normal saline; aCSF, artificial cerebrospinal fluid; MIN, minocycline; PDTC, pyrrolidine dithiocarbamate.

nificantly after PVN administration of PDTC or MIN (Fig. 5B).

PVN administration of MIN or PDTC attenuated oxidative stress in PVN

Real-time PCR was done for quantification of gp91^{phox} mRNA in PVN. It was exhibited that ANG II-induced hypertensive rats had more gp91^{phox} mRNA expression in PVN when compared to control ones (Fig. 6). PVN administration of MIN attenuated partly but not completely gp91^{phox} mRNA expression in PVN of ANG II-induced hypertensive rats. PVN administration of PDTC did not significantly suppress PVN gp91^{phox} mRNA expression of ANG II-induced hypertensive rats (Fig. 6).

PVN administration of MIN or PDTC modulated neurotransmitters in PVN

GABA is an inhibitory neurotransmitter which can lower arterial pressure. Glu is an excitatory neurotransmitter, which can bind to ionotropic receptors to amplify the excitability of neurons. The relative expression of Glu in PVN of ANG II-induced hypertensive rats was increased significantly when compared to control rats (Fig. 7A), and the relative expression of GABA in the PVN of ANG II-induced hypertensive rats was reduced significantly in comparison with control rats (Fig. 7B). After PVN administration of PDTC or MIN, the relative expression of Glu in PVN was significantly decreased when compared with ANG II-induced hypertensive rats treated with aCSF (Fig. 7A). And PVN administration of MIN or PDTC did not affect the relative expression of GABA in PVN of ANG II-induced hypertensive rats significantly (Fig. 7B). The relative expression of NE in PVN of ANG II-induced hypertensive rats was significantly increased in comparison with control ones (Fig. 7C), which was decreased by the treatment with PVN infusion of MIN or PDTC.

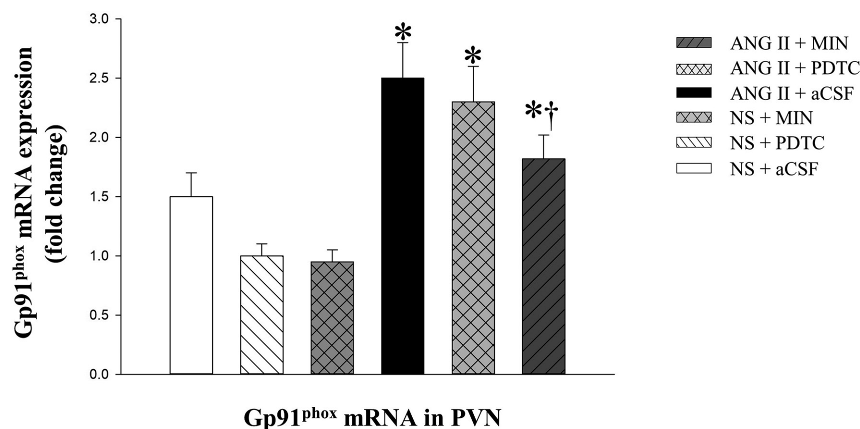

Fig. 6. Effects of paraventricular nucleus infusion of minocycline or pyrrolidine dithiocarbamate on mRNA expression of gp91^{phox} (source of reactive oxygen species production) in the paraventricular nucleus of angiotensin II-induced hypertensive rats.
Group data for gp91^{phox} mRNA expression in the PVN. Paraventricular nucleus infusion of minocycline partly but not completely suppressed gp91^{phox} mRNA expression in the paraventricular nucleus of angiotensin II-induced hypertensive rats. Paraventricular nucleus infusion of pyrrolidine dithiocarbamate did not significantly suppress gp91^{phox} mRNA expression in the paraventricular nucleus of angiotensin II-induced hypertensive rats. $^{*}P < 0.05$ vs. control rats (NS + aCSF or NS + MIN/PDTC); $^{\dagger}P < 0.05$ ANG II + MIN/PDTC vs. ANG II + aCSF.
PVN, paraventricular nucleus; NS, normal saline; aCSF, artificial cerebrospinal fluid; MIN, minocycline; PDTC, pyrrolidine dithiocarbamate; ANG II, angiotensin II; Gp91^{phox}, subunits of (NAD(P)H) oxidase.

PVN administration of MIN or PDTC attenuated NF-κB p65 in PVN

NF-κB p65 activity in PVN of ANG II-induced hypertensive rats increased when compared to control groups. PVN infusion of MIN or PDTC lowered increasement of NF-κB p65 of ANG II-induced hypertensive rats (Fig. 8).

Discussion

The findings of the current study are: 1) microglia were activated in the PVN, where they may be a source of proinflammatory cytokines, ROS and PGE_2, and imbalance of neurotransmitters that stimulate neurohumoral excitation in ANG II-induced hypertension; 2) PVN infusion of MIN, which is an inhibitor of activated microglia, reduced PVN proinflammatory cytokines, CSF PGE_2, NF-κB p65, excitatory neurotransmitters, and partly attenuated ROS, and therefore suppressed sympathetic overactivity and MAP in ANG II-induced hypertension; 3) PVN infusion of PDTC, which is an inhibitor of NF-κB, decreased microglial activation in the PVN of hypertension, accompanied by reduction of PVN proinflammatory cytokines, CSF PGE_2, NF-κB p65, and excitatory neurotransmitters, and attenuated sympathetic overactivity and MAP in ANG II-induced hypertension; 4) the results that MIN suppressed NF-κB p65 and PDTC attenuated microglia in the PVN of ANG II-induced hypertensive rats indicate that activated microglia may affect the pathophysiological process of hypertension by NF-κB pathway.

Hypertension is characterized by high arterial pressure. Large evidences have pointed to angiotensin and sympathetic nervous system as principal elements associated with elevated arterial pressure (Fisher and Paton 2012). As the major integrative center of the forebrain, the PVN is the best understood source of presympathetic neurons that directly innervate the rostral ventrolateral medulla and the intermediolateral cell column of the spinal cord, which contains the preganglionic sympathetic neurons (Shen et al. 2018). Previous studies have found that activated renin-angiotensin system or ROS in PVN contributes to sympathoexcitation in heart failure and hypertension, and that proinflammatory cytokines induced by angiotensin in the PVN also participate in the sympatho-mechanism in hypertension (Su et al. 2014).

As messengers among immune cells and inflammation, proinflammatory cytokines interact with different tissues and organs, and assume new characters in addition to previously known functions. They are capable of acting as neuromodulators within neurons. Studies about proinflammatory cytokines in the peripheral system are abundant. Among these, plasma proinflammatory cytokines have been demonstrated to upregulate blood pressure by affecting vascular function and endothelium-derived factors (Yu et al. 2019). It was reported that increased proinflammatory cytokines in the PVN contribute to the activation of sympathetic drive (Yu et al. 2022), but knowledge surrounding the effect of brain proinflammatory cytokines on neurohumoral excitation is still very limited.

We have found that PVN proinflammatory cytokines in ANG II-induced hypertensive rats have pressor effects by stimulating sympathetic drive, which further promotes the release of other neurohumoral factors (Kang et al. 2014b; Yu et al. 2019). PVN administration of PDTC suppressed activated microglia, decreased PVN levels of proinflammatory cytokines, and PVN NF-κB p65, impaired CSF PGE_2, and depressed circulating NE and part of circulating ANG II. All these results suggest that inflammation caused by

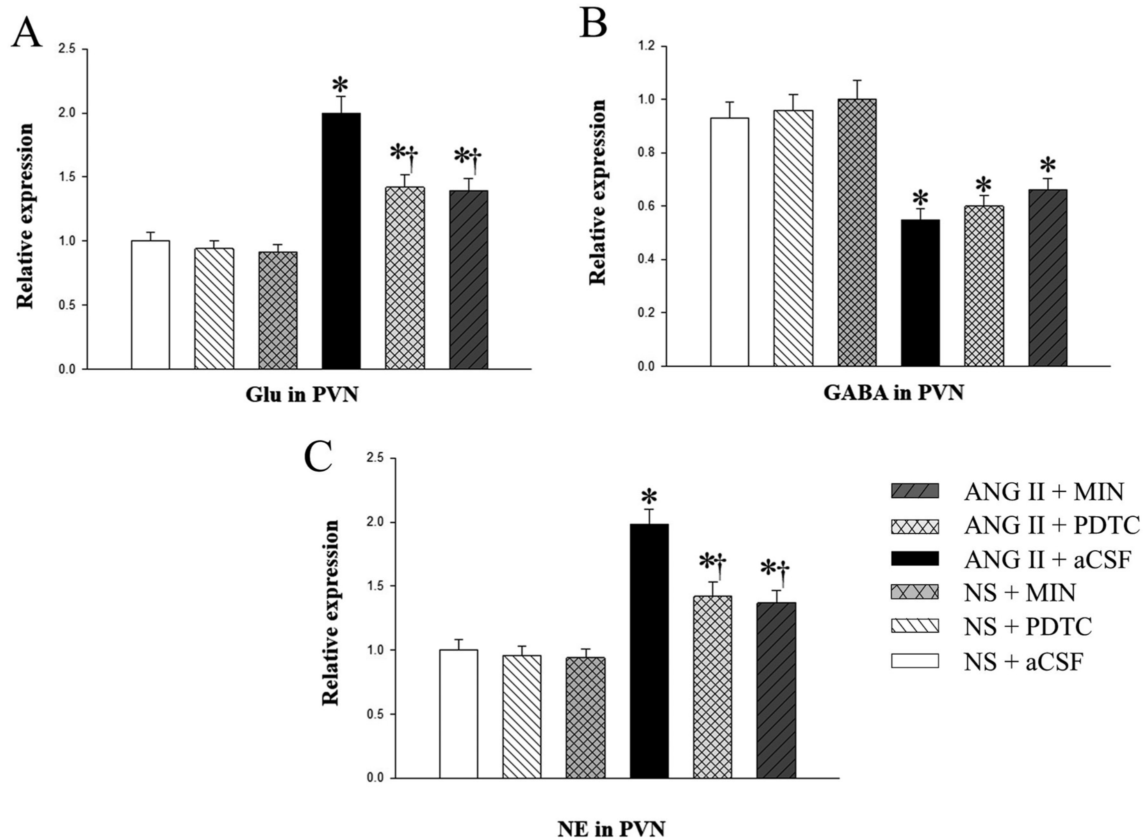

Fig. 7. Effects of paraventricular nucleus infusion of minocycline or pyrrolidine dithiocarbamate on neurotransmitters in paraventricular nucleus of angiotensin II-induced hypertensive rats.
A. Group data for the relative expression of glutamate (Glu) in the PVN. $^*P < 0.05$ vs. control rats (NS + aCSF or NS + MIN/PDTC); $^\dagger P < 0.05$ ANG II + MIN/PDTC vs. ANG II + aCSF. B. Group data for the relative expression of γ-aminobutyric acid (GABA) in the PVN. Paraventricular nucleus infusion of minocycline or pyrrolidine dithiocarbamate did not significantly affect the relative expression of GABA in the PVN of angiotensin II-induced hypertensive rats. $^*P < 0.05$ vs. control rats (NS + aCSF or NS + MIN/PDTC). C. Group data for the relative expression of norepinephrine (NE) in the PVN. $^*P < 0.05$ vs. control rats (NS + aCSF or NS + MIN/PDTC); $^\dagger P < 0.05$ ANG II + MIN/PDTC vs. ANG II + aCSF.
Glu, glutamate; GABA, γ-aminobutyric acid; NE, norepinephrine; PVN, paraventricular nucleus; NS, normal saline; aCSF, artificial cerebrospinal fluid; MIN, minocycline; PDTC, pyrrolidine dithiocarbamate; ANG II, angiotensin II.

activated microglia in PVN are devoted to neurohumoral excitation by NF-κB in ANG II-induced hypertension. The excitability of sympathetic preneurons in the PVN is fine-tuned by excitatory (Glu and NE) and inhibitory (GABA) neurotransmitters. Impaired GABA synaptic input or increased Glu synaptic input results in hyperactivity of sympathetic presynaptic cells, increased sympathetic efferent activity (Zhou et al. 2019) and increased blood pressure. In this study, PVN NE and Glu, as well as plasma NE, were elevated, and PVN GABA was decreased in ANG II-induced hypertensive rats. The present study demonstrated that both MIN and PDTC suppressed the high expression of excitatory neurotransmitters (Glu and NE) in ANG II-induced hypertension, but we did not find their effect on the change of inhibitory neurotransmitters (GABA) in ANG II-induced hypertension.

Microglia, as the important immunoreactive cells in the CNS, contribute to the inflammatory response in the brain. Microglia spread over CNS, including the PVN, and comprise 20% of the total glial cells in brain. As the "resident immune cells" of the brain, microglial cells produce a wide variety of inflammatory products, including proinflammatory cytokines, ROS and PGE_2 (Dringen 2005). As subunit of NAD(P)H oxidase, the activity of $gp91^{phox}$ in neurons is a major source of superoxide anion leading to increased oxidative stress, sympathetic excitability, and incremental blood pressure (Campos et al. 2015). Particularly interesting is the fact that NAD(P)H oxidase activity plays a major part in synthesis of TNF-α, IL-1β and PGE_2 by the microglia (Dringen 2005). The expression of $gp91^{phox}$ indirectly reflect the NAD(P)H oxidase activity. The present study showed PVN administration of MIN decreased proinflammatory cytokines in the PVN, and partly but not completely attenuated plasma ANG II and PVN ROS in ANG II-induced hypertension. Thus, activated microglia is a potential source of proinflammatory cytokines and ROS in brain of hypertensive rats.

NF-κB is a key factor which plays a central regulatory

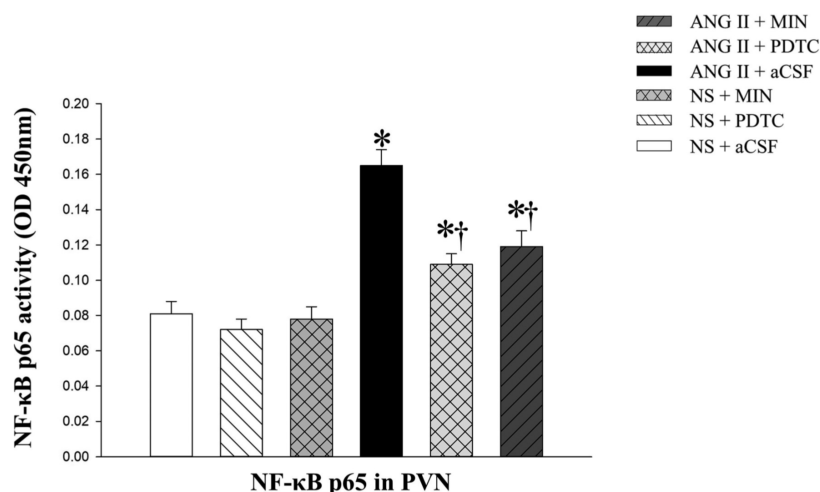

Fig. 8. Effects of paraventricular nucleus infusion of minocycline or pyrrolidine dithiocarbamate on nuclear factor-kappa B p65 activity in paraventricular nucleus of angiotensin II-induced hypertensive rats.
Group data for nuclear factor-kappa B (NF-κB) p65 activity in the PVN. *$P < 0.05$ vs. control rats (NS + aCSF or NS + MIN/PDTC); $^{\dagger}P < 0.05$ ANG II + MIN/PDTC vs. ANG II + aCSF.
NF-κB, nuclear factor-kappa B; PVN, paraventricular nucleus; NS, normal saline; aCSF, artificial cerebrospinal fluid; MIN, minocycline; PDTC, pyrrolidine dithiocarbamate; ANG II, angiotensin II.

part in inflammatory response. It is composed of p50 and p65 subunit. They are in all cell types in the nervous system, of which p65 subunit contains transcriptional activation region at the C-terminal of protein. The transcriptional activation region can specifically bind to the κB sequence binding site on nuclear DNA to initiate transcription of a series of genes. Activated NF-κB is the major regulator facilitating the synthesis of several different injury-responsive cytokines in neurons, including proinflammatory cytokines, which contribute to sympathoexictation in hypertension (Hochrainer et al. 2013).

In this study, NF-κB p65 activity was measured by ELISA, and the result showed that the level of p65 in ANG II-induced hypertensive rats was increased. Microglia inhibitor MIN and NF-KB inhibitor PDTC notably reduced the activity of p65. Immunofluorescence showed that there were activated microglia in the PVN region in ANG II-induced hypertension rats. The stubby appearance of activated microglia became slender (Dworak et al. 2012) after PVN administration of PDTC. All these results demonstrated that the promoting effect of activated microglia on the pathophysiological process of hypertension may be related to the upregulation of NF-κB p65 expression. MIN alleviating the p65 activity indicated that MIN may suppress the activation of microglia by inhibiting the activation of NF-κB, thereby impairing the proinflammatory cytokines and ROS. PDTC can inhibit the activation of microglia, and once again proved that inhibition of the activity of NF-κB can reduce the activation of microglia, thus attenuating the progress of high blood pressure. The findings in this research may offer new strategies to hypertension.

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Author Contributions

Y.-M.K. and Z.-M.Y. designed the study. X.-J.L. and X.-J.Y. performed all experiments. Y.-M.K., Z.-M.Y., X.-J. L. and X.-J.Y. also performed the data analysis and drafted the manuscript. Y.-M.K., X.-J.Y., X.-J.L. and Y.-K.S. participated in data analysis. Y.-M.K., Z.-M.Y., X.-J.L., X.-J. Y., Y.-K.S., and L.-X.Y. critically revised the manuscript. All authors reviewed the final manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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